Reports

Protease Inhibitor Content of Human Dietary Samples

Paul C. Billings, Matthew P. Longnecker, Matthew Keary, and Phillip R. Taylor

Abstract

A large body of experimental work has revealed that protease inhibitors (PI) are highly effective suppressors of carcinogenesis. Little is known about the level of PI activity in the diet of the US population. In the present study, we assayed the levels of PI activity in dietary samples from 31 free-living subjects who saved duplicate portions of all foods consumed over two 24-hour periods, six months apart. The majority of samples (90%) contained detectable PI activity; 82% contained trypsin inhibitory activity; 61% contained chymotrypsin inhibitory activity. Of those samples containing chymotrypsin inhibitory activity, 87% also contained trypsin inhibitory activity. The median concentration of soluble chymotrypsin inhibitory activity present in these samples was 6.5 μg/g food (range 0–150 μg/g food), whereas the median concentration of soluble trypsin inhibitory activity was 14.5 μg/g food (range 0–465 μg/g food). We conclude that a) human diet samples contain both chymotrypsin and trypsin inhibitory activity, b) the levels of PI in some of these samples was similar to that found to be anticarcinogenic in animal studies, and c) due to the large within-subject variation in PI intake, assessment of long-term dietary intake in epidemiological studies will be necessary to accurately classify subjects according to PI intake.

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Introduction

Epidemiological data suggest that nutritional factors play an important role in the etiology of cancer at several different sites. Further, it appears that consumption of certain foods and nutrients derived from plants is associated with a decreased risk of cancer (1–9). Mills and co-workers (4) recently reported that intake of protein products derived from legumes is

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inversely associated with the risk of pancreatic cancer. Phillips (5) examined the relationship between the risk of colon cancer and intake of vegetarian protein products; the relative risk for those with frequent consumption was half that compared with subjects consuming these products less often. In a recent case-control study of colon and rectal cancer, subjects who frequently consumed tofu, soybeans, or lentils had a relative risk of colon cancer half that of those consuming these products less often (9). Japanese subjects consuming soybean paste soup daily have been found to have a significantly reduced risk of cancers of all types compared with those consuming this soup less often (6), whereas other studies have reported that subjects consuming miso soup frequently had a risk of colon cancer half of those consuming this soup less often (8). These data suggest that legumes, such as soybeans, deserve further investigation as cancer chemopreventive agents.

Legumes are known to contain relatively high concentrations of protease inhibitors (PI) (10). PIs from various sources have been shown to have strong anticarcinogenic activity (11–17). As a group, inhibitors of chymotrypsin appear to be the most effective suppressors of carcinogenesis *in vitro* (13). However, certain PIs, such as soybean trypsin inhibitor (SBTI), which is a strong trypsin inhibitor but a very weak chymotrypsin inhibitor (10), will suppress the enhancement of radiation transformation by tumor promoters (12,13). Therefore, both trypsin and chymotrypsin inhibitors deserve evaluation as human cancer chemopreventive agents.

Although previous studies have determined the levels of trypsin inhibitory activity (TIA) present in many foods (18–20), relatively little information is currently available regarding the levels of PI activity present in the diet or the intake of PIs by individuals living in the United States. Further, although some data predicting average PI intake have been reported (18), the variation between individuals in PI intake has not been examined. Such information would be useful in planning and assessing epidemiological evaluations of associations between PI intake and cancer risk. In the current study, we have assayed for PI activity dietary samples obtained from free-living subjects that participated in a study of dietary selenium intake.

Materials and Methods

Chemicals

Chymotrypsin and trypsin were obtained from Sigma (St. Louis, MO). The trypsin substrate Bz-Arg-MCA and chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-MCA (21) were obtained from Peninsula Laboratories (Belmont, CA) and made up as 1 mg/ml stock solutions in spectrograde DMSO and stored at 4°C. Trypsin and chymotrypsin were prepared in 0.001N HCl at a concentration of 1 mg/ml.

Food Samples

Two groups of samples were analyzed. The first group was obtained from free-living subjects enrolled in the Selenium Safety Evaluation, a study sponsored by the National Cancer Institute (Bethesda, MD). During each 24-hour collection period, subjects saved duplicate portions of all foods consumed in a single container that was kept refrigerated or frozen. Each subject maintained a diet record listing the kinds and amounts of foods consumed (and saved) during the collection periods. In the laboratory, the specimens were thawed (if necessary) and homogenized with a food processor; aliquots were frozen and lyophilized. A subset of the lyophilized samples were selected for use in the current study. Samples from two 24-hour collection periods, six months apart, for each of 31 subjects were assayed for PI activity. A second group of food samples was gathered by three subjects over a one-day period. Each subject selected three meals: one meal with a low level of TIA, one

meal with a moderate level of TIA, and one meal with a high level of TIA. The level of PI activity present in the meals consumed by these subjects was controlled by reducing or increasing the levels of specific dietary components known to contain high levels of PI activity (such as legumes) (10,18–20). Each subjects' samples from the three meals were refrigerated until they were homogenized and aliquoted.

Sample Preparation and Analysis

One-half gram of food sample was weighed into a 15-ml plastic centrifuge tube, and 10 ml of distilled water were added. The sample was disrupted in a Brinkman polytron homogenizer [two cycles (top speed, 1 min, 10 min apart)] and centrifuged at 2,000 *g* for 10 minutes to pellet insoluble material; the supernatant fraction was used as the PI source. Each sample was analyzed for its ability to inhibit chymotrypsin and trypsin activity. Chymotrypsin activity was assayed in 50 mM Tris-HCl (pH 7.8), 10 mM $CaCl_2$ (Reaction Buffer) using the fluorescent substrate Suc-Ala-Ala-Pro-Phe-MCA (11). The release of the fluorescent reporter group MCA (4-*methyl*-coumaryl-7-amide) was determined spectrofluorometrically at excitation and emission wavelengths of 380 and 460 nm, respectively (11). To assay for PI activity, aliquots of 10, 25, 50, 100, and 200 μl from each food sample extract were incubated with 0.25 μg of chymotrypsin or trypsin for five minutes in reaction buffer; then substrate was added and the reaction was directly monitored in a Perkin Elmer LS 5 spectrofluorometer for five minutes. TIA was determined in an analogous fashion, except that Bz-Arg-MCA was used as substrate. Analytical blanks, containing reaction buffer, food sample, and substrate (but lacking trypsin or chymotrypsin), were also run for each sample; no substrate hydrolysis was observed in these samples. In addition, no quenching of the fluorescent reporter group (MCA) by the food samples was observed. To determine the intra-assay coefficient of variation (CV), samples from four subjects were repeatedly analyzed. The CVs for trypsin and chymotrypsin inhibition assays were both $<6\%$.

Amount of PI Present in Food Samples

We have estimated the amount of PI per gram of food sample. These calculations were made by assuming the following three things. 1. One PI molecule combines with one molecule each of trypsin or chymotrypsin; for example, SBTI is known to interact with one molecule of trypsin, whereas the Bowman Birk Inhibitor (BBI) interacts with one molecule of chymotrypsin and trypsin (10). 2. At 20% inhibition, the vast majority of PI present in the sample is complexed with the protease. The association constant of protease inhibitors such as BBI for trypsin and chymotrypsin is high (10). 3. The mass of the PI is similar to that of the protease: the molecular mass of BBI and SBTI is about 8 and 20 kilodaltons, respectively (10). To perform these calculations, we first determined the amount of food sample extract required to inhibit protease activity 20%. Next, the quantity of PI present was determined using the following equation:

$$[0.20/\text{Volume (in ml) of extract to yield 20\% inhibition}] \times 0.25\ \mu\text{g of enzyme} \times 20\ \text{ml/g food} = \mu\text{g PI/g food}$$

Results

The level of PI activity present in the 62 dietary samples from subjects enrolled in the Selenium Safety Evaluation Study are summarized in Table 1. A sample was considered to contain PI activity if 200 μl or less of sample extract inhibited protease activity at least 20%. The majority of samples examined (90%) contained PI activity (Table 1). TIA was present in 82% of the samples, whereas chymotrypsin inhibitory activity (CTIA) was observed in

Table 1. Protease Inhibitor (PI) Activity Present in Food Samples From 31 Free-Living Subjects[a]

Subject	Sample	Volume of Extract to Yield 20% CTIA,[b] μl	CTIA,[c] μg/g Food	Volume of Extract to Yield 20% TIA,[b] μl	TIA,[c] μg/g Food
1	A	>200	0	20	50
1	B	154	6.5	6	170
2	A	>200	0	21	48
2	B	>200	0	143	7
3	A	154	6.5	16	62
3	B	>200	0	>200	0
4	A	>200	0	32	31
4	B	>200	0	133	7.5
5	A	154	6.5	105	9.5
5	B	>200	0	182	5.5
6	A	11	88	23	44
6	B	>200	0	14	69
7	A	20	50	71	14
7	B	118	8.5	105	9.5
8	A	37	27	>200	0
8	B	143	7	13	75
9	A	7	150	67	15
9	B	>200	0	11	87
10	A	111	9	48	21
10	B	105	9.5	105	9.5
11	A	182	5.5	>200	0
11	B	154	6.5	33	30
12	A	100	10	2	465
12	B	125	8	56	18
13	A	118	8.5	83	12
13	B	83	12	77	13
14	A	167	6	143	7
14	B	>200	0	111	9
15	A	77	13	3	375
15	B	125	8	43	23
16	A	>200	0	21	47
16	B	200	5	16	62
17	A	91	11	16	62
17	B	>200	0	111	9
18	A	>200	0	125	8
18	B	154	6.5	133	7.5
19	A	125	8	2	445
19	B	>200	0	>200	0
20	A	100	10	154	6.5
20	B	83	12	182	5.5
21	A	>200	0	37	27
21	B	>200	0	38	26
22	A	>200	0	12	83
22	B	71	14	2	465
23	A	>200	0	>200	0
23	B	33	30	83	12
24	A	>200	0	83	12
24	B	83	12	3	385
25	A	111	9	>200	0
25	B	100	10	6	164

(continued)

From 31 Free-Living

e of t to 20% ,[b]	TIA,[c] μg/g Food
20	50
6	170
21	48
43	7
16	62
00	0
32	31
33	7.5
05	9.5
82	5.5
23	44
14	69
71	14
05	9.5
00	0
13	75
67	15
11	87
48	21
05	9.5
00	0
33	30
2	465
56	18
83	12
77	13
43	7
11	9
3	375
43	23
21	47
16	62
16	62
11	9
25	8
33	7.5
2	445
200	0
154	6.5
182	5.5
37	27
38	26
12	83
2	465
200	0
83	12
83	12
3	385
200	0
6	164

(continued)

Table 1. *(Continued)*

Subject	Sample	Volume of Extract to Yield 20% CTIA,[b] μl	CTIA,[c] μg/g Food	Volume of Extract to Yield 20% TIA,[b] μl	TIA,[c] μg/g Food
26	A	71	14	>200	0
26	B	>200	0	100	10
27	A	63	16	22	46
27	B	167	6	42	24
28	A	200	5	33	30
28	B	100	10	11	88
29	A	133	7.5	133	7.5
29	B	>200	0	>200	0
30	A	>200	0	>200	0
30	B	91	11	>200	0
31	A	>200	0	>200	0
31	B	>200	0	67	15

a: Dietary samples were extracted as described in **Materials and Methods** and analyzed for their ability to inhibit trypsin and chymotrypsin.

b: Volume (μl) of sample extract required to reduce protease activity 20%. CTIA, chymotrypsin inhibitory activity; TIA, trypsin inhibitory activity.

c: Quantity of PI present in samples is expressed as μg PI/g food. Value of zero indicates that 200 μl of that sample extract inhibited protease activity <20%.

61%. CTIA and TIA tended to be present in the same sample; 87% of those samples containing CIA also contained TIA, whereas 65% of the samples containing TIA also contained CIA. The median level of soluble CTIA in the 62 samples was 6.5 μg/g food (range 0–150 μg/g food), whereas the median level of TIA in the samples was 14.5 μg/g food (range 0–465 μg/g food) (Table 1).

The quantity of PI present in each gram of sample was estimated as described in **Materials and Methods.** We next estimated the total amount of PI activity consumed on each day by multiplying the weight of food consumed by each subject by the concentration of PI present. We then calculated the average amount of PI in the diet each day for each of the 31 subjects. The median amount of soluble CTIA consumed daily was 1.6 mg (range 0–31.1 mg) and for TIA was 4.6 mg daily (range 0–127.9 mg). The distribution of average daily PI intake is shown in Figure 1. The average daily intake of TIA was greater than that of CTIA (Figure 1). These results may reflect the fact that the levels of TIA present in these foods is higher or that TIA is more resistant to denaturation by cooking (or other methods of food preparation) than is CTIA.

A second group of dietary samples, obtained from individuals in our laboratory, were also assayed for PI activity (Table 2). Because food composition tables of PI content are not complete, the total amount of PI present in these meals could be evaluated only crudely. The presence of relatively higher levels of TIA in the second and third samples and submitted by each subject demonstrated that the assay yielded an inhibitor content consistent with the anticipated levels of TIA present in several of the foods (10,18–20) in the sample, thus informally validating the assay.

These studies indicate that a substantial variation exists in the average quantities of water soluble PI activity present in a US diet. We also evaluated the variation in daily intake within and between individuals. The intraclass correlation coefficient for soluble TIA was 0.07 (not significant) and for chymotrypsin was 0.10 (not significant). In other words, of the total

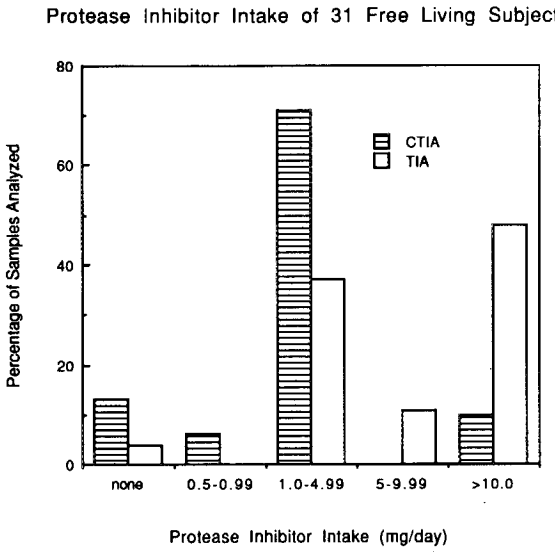

Figure 1. Histogram indicating amount of protease inhibitor activity consumed daily by subjects participating in Selenium Safety Evaluation Study. CTIA, chymotrypsin inhibitory activity; TIA, trypsin inhibitory activity.

variation in TIA and CTIA among the 62 specimens, most was due to the variation in diet within subjects.

Discussion

Although recognition of the influence of diet on the occurrence of cancer is widespread, few nutrients have been shown to be effective as preventive agents in humans. PIs have been shown to be effective suppressors of carcinogenesis in several experimental systems; these studies suggest that it would be of interest to evaluate the relation between the intake of PIs and cancer risk in humans. For example, we have reported that the dietary addition of BBI will suppress dimethylhydrazine-induced colonic adenomas in mice (17). In more recent studies, we have found that diets containing 0.01%–0.1% pure BBI (100–1,000 μg PI/g dry lab chow) will inhibit the formation of adenocarcinomas of the colon (15). The maximal levels of PI activity present in the dietary samples assayed in our samples was 465 μg/g food for TIA. (This number may reflect a lower estimate of the total amount of PI activity actually present in these samples, because some PI activity may not have been completely solubilized.) Therefore, the dietary levels of PI present in some of the dietary samples analyzed in the current study were in the same range as the levels shown to inhibit experimental colon cancer in mice (15,17).

Most of the total variation in PI intake in this study was due to that within subjects. This pattern of variation is similar to that for cholesterol, where there is also a great variation in a subject's intake from day to day (22). The implication for epidemiological studies is that data on long-term dietary intake (e.g., a period of 2 or more weeks) will be necessary to accurately classify subjects according to PI intake. The spread in average PI intake, as shown in Figure 1, suggests that there is enough variation to make worthwhile an examination of the relation of intake to the risk of cancer in epidemiological studies.

An important consideration in analyzing the PI content of dietary samples is the extraction method used to solubilize the PIs present in a given sample. In the human digestive tract, food enters the stomach and is subjected to an acidic environment and then moves to the small intestine where the contents are in an aqueous environment at pH 6.4–7.5 (23). In the present study, we utilized a neutral pH extraction system, because we feel this approximates the pH in the small intestine. Previous studies utilized an alkaline extraction procedure (18,19) to analyze PI present in foods. Although this may be a more efficient system for

Table 2. Protease Inhibitor (PI) Activity in Dietary Samples From Laboratory Subjects[a]

Subject	Sample	Volume of Extract to Yield 20% CTIA,[b] µl	CTIA, µg/g Food	Volume of Extract to Yield 20% TIA,[b,c] µl	TIA, µg/g Food
32	A	32	31	143	7
32	B	36	28	26	38
32	C	25	40	5	182
33	A	56	18	200	5
33	B	24	42	0	0
33	C	19	52	43	23
34	A	43	23	143	7
34	B	42	24	143	7
34	C	18	56	2	455

a: Foods in these samples were selected so that the first sample collected by each subject had a low PI content, whereas the second and third samples contained a higher inhibitor content. Foods present in each sample were as follows: 32 A: hamburger with bun (1), french fries (large), cole slaw (½ cup); 32 B: scrambled eggs (¾ cup), chipped beef (1 cup), white biscuit (1), strawberries (10), half & half (1 tbsp); 32 C: spinach (raw) (1 cup), romaine (½ cup), chickpeas (¾ cup,) kidney beans (¼ cup), three bean salad (¼ cup), sliced beets (½ cup), sliced carrots (raw; ⅛ cup), egg (1), tunafish (¼ cup), red cabbage (raw; ¼ cup), green pepper (raw; 2 rings), green olives (3 small), jalapeño (½ small), cheddar cheese (¼ cup), broccoli (3 flowerettes); 33 A: Chicken McNuggets (9), french fries (large), cole slaw (4 oz), sauce for chicken (sweet and sour); 33 B: fried potatoes (½ cup), grits (⅔ cup), bacon (3 strips), sausage (3 links), pancakes (3–4 in. in diam), cinnamon apple muffin (1), biscuit (1), butter (2 pats), syrup (2 oz); 33 C: green salad (1.16 lb) with ranch dressing, sesame sticks (4), beets (4 slices), tofu (1-in. square chunk), carrots (⅛ cup), artichoke hearts (1), chickpeas (½ cup), broccoli (2 heads), mushrooms (⅛ cup), bean sprouts (⅛ cup), lettuce (½ cup), spinach (3 leaves), red cabbage (⅒ cup), green pepper (2 slices), cauliflower (1 flower), red kidney beans (⅛ cup); 34 A: Jello & sparse fruit (1.25 cup), spinach (⅛ cup), broccoli (½ cup), carrots (⅓ cup), cauliflower (½ cup), red cabbage (¼ cup); 34 B: french fries (1½ cup), cole slaw (⅔ cup), hamburger (1), roast beef sandwich (1); 34 C: eggs (2), fried potatoes (1 cup), three bean salad (1½ cups), half & half (1 tbsp), toast (2 slices).

b: Volume (µl) of sample extract required to inhibit chymotrypsin or trypsin activity 20%.

c: Value of 0 indicates that 200 µl of that sample extract inhibited protease activity <20%.

solubilizing TIA, it is significantly different from the conditions present in the human digestive tract and may not be a true reflection of the PI activity that is solubilized in humans. Thus, with respect to the occurrence of disease in humans, the result of the assay with alkaline extraction may not reflect the PI activity that is biologically relevant. For epidemiological purposes, food composition tables may be more relevant if the PI activity present in these foods is extracted in a manner that simulates the conditions in the human digestive system. This should give the most physiologically relevant value for PI activity present in dietary samples.

It has been suggested that high levels of PIs in the diet may lead to undesirable side effects such as pancreatic carcinogenesis or reduced weight gain in growing animals (24,25). However, several lines of evidence suggest that dietary levels of PI sufficient to suppress carcinogenesis may not have adverse effects. First, there are human populations, such as the Japanese and Seventh-Day Adventists, who consume high levels of dietary PIs and have no increased risk of pancreatic cancer (in fact, there is a lower than normal risk of pancreatic cancer in these populations) (1–9). Second, in animal experiments conducted in our laboratory, no undesirable side effects, including pancreatic changes, decreased growth rate, or a decline in general health, have been observed in animals maintained on high levels of anticarcinogenic dietary PIs for their entire life span (13–15,17).

Cancer prevention via dietary means may well be the most cost-effective approach toward controlling this disease. Work from several laboratories has demonstrated that PIs are effective suppressors of carcinogenesis; these studies suggest that PIs may be useful human cancer chemopreventive agents (16). The results from the current study suggest that sufficient variation exists in PI intake so that further epidemiological studies are merited to examine the relationship between PI intake and cancer incidence. A comprehensive nutrient data base for PIs would make possible an assessment of the effect of these substances on cancer risk in humans.

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